Short communication

Requirements for mouse mammary tumour virus internalization in mouse mammary epithelial cells

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Methylamine, a lysosomotropic alkalinizing agent, blocked mouse mammary tumour virus (MMTV) infection in normal mouse mammary epithelium, suggesting that internalization and acidification are necessary for cell penetration. This mechanism was further supported by the fact that intact MMTV induced the translocation of its cellular binding protein from the plasmalemma to the microsomes; however, isolated gp52, the MMTV envelope protein that binds this receptor, did not redistribute the binding protein. These data suggest that either another viral component, in addition to gp52, is needed for cell entry or that internalization requires receptor aggregation, which only the multivalent viral envelope can induce.

Mouse mammary tumour virus (MMTV) is an enveloped retrovirus that was postulated to require endocytosis and acidification for successful infection (Redmond et al., 1984). Recently, this laboratory identified a mammary epithelial membrane protein that binds MMTV (Bolander & Blackstone, 1991). Because its distribution coincided with the target tissues for MMTV and because receptor antibodies blocked infection in vitro (Bolander, 1993a), this receptor might be expected to mediate the cell entry of MMTV. The actual ligand for this cellular receptor is gp52, an MMTV envelope protein; however, although isolated gp52 binds specifically and with high affinity to this protein, gp52 does not induce its internalization (Bolander, 1994), suggesting that MMTV might penetrate the cell at the surface. The discrepancy between these two studies might be explained by a difference in the tissues used: the original investigation employed mink lung cells, while the more recent work involved mammary epithelium. It is known that some viruses utilize different mechanisms of cell entry in different cell types (Marsh & Pelchen-Matthews, 1993); therefore, this study was initiated to examine more thoroughly the route of MMTV infection in the mammary epithelium, the natural target for the MMTV.

Ovine prolactin (oPRL-17) was kindly provided by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, Md., USA), and crystalline porcine insulin (lot 615-08E-220) was a gift from Eli Lilly & Co. (Indianapolis, Ind., USA). Cortisol, T3, retinoic acid, ammonium chloride, chloroquine, methylamine, bovine α-lactalbumin, bovine serum albumin and HEPES were purchased from Sigma. Medium 199 with Hanks’ balanced salt solution was obtained from GIBCO. Polyethylene glycol 6000 was from Eastman Kodak, and dextran T500 and Sephadex G-75 were purchased from Pharmacia LKB. Na125I (carrier-free) and [32P]orthophosphoric acid (8500–9120 Ci/mol) were obtained from New England Nuclear, and UDP-[6-3H]galactose (18.9 mCi/mmol) was from Amersham.

Virgin mice (C3H/HeN MMTV+ and MMTV−) were purchased from the National Cancer Institute (Bethesda, Md., USA). Mammary gland explants were prepared as previously described (Juergens et al., 1965). The explants were cultured on siliconized lens paper in medium 199 containing 20 mM-HEPES (pH 7.5), insulin (1 μg/ml), cortisol (10 ng/ml), prolactin (1 μg/ml) and T3 (65 pg/ml), unless otherwise noted. The tissue was incubated under air at 37 °C and the medium was changed daily.

α-Lactalbumin was assayed in mammary explants after a 3 day culture in hormones with or without ammonium chloride (10 mM), chloroquine (50 μM) or methylamine (1 mM). Enzymatic activity was measured by a modification (Ono & Oka, 1980) of the method of Fitzgerald et al. (1970) using bovine α-lactalbumin as standard.

The ability of MMTV to infect mammary epithelium was assayed by the method of Bolander (1993b). Briefly, tissue from MMTV− mice was exposed to MMTV in culture and then induced to express MMTV RNA, which was measured by dot blot as previously described.
Table 1. Effect of lysosomotropic amines on \( \alpha \)-lactalbumin induction

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Amine concn</th>
<th>( \alpha )-Lactalbumin activity (ng/100 mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin and cortisol</td>
<td></td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Insulin, cortisol and prolactin</td>
<td>10 mM</td>
<td>618 ± 66</td>
</tr>
<tr>
<td>Insulin, cortisol, prolactin and ammonium chloride</td>
<td>50 ( \mu M )</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>Insulin, cortisol, prolactin and chloroquine</td>
<td>1 mM</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>Insulin, cortisol, prolactin and methylamine</td>
<td></td>
<td>517 ± 59†</td>
</tr>
</tbody>
</table>

* The results represent the mean ± one standard error from six independent experiments.
† Not significantly different from the insulin, cortisol and prolactin control by the analysis of variance.

(Muñoz & Bolander, 1989); the expression of endogenous MMTV RNA was so low that it did not interfere with this assay. The results were quantified by densitometry of the autoradiograms, which contained internal standards.

The cellular binding protein for MMTV was measured on plasma membranes and microsomes. After culture with either gp52 or intact MMTV, mammary epithelial cells were fractionated by the method of Brunette & Till (1971). Briefly, the cells were homogenized and added to centrifuge tubes containing a discontinuous gradient composed of dextran T500 and polyethylene glycol; plasma membranes formed at the interface and microsomes remained in the supernatant. Cross-contamination averaged less than 2% (Brunette & Till, 1971). After recovery, radio-iodinated gp52 was incubated with each fraction (Bolander, 1994). Protein was determined by the Lowry method using BSA as standard, and binding data were analysed by the method of Scatchard (1949).

The phosphorylation of MMTV binding protein was measured by equilibrating the epithelium with \([^{32}P]\)orthophosphoric acid, adding gp52 or MMTV, and immunoprecipitating the receptor after 5 min (Bolander, 1994). In these and all other experiments, gp52 was used at a concentration of 0.1 \( \mu g/\)ml and MMTV at \( 2 \times 10^8 \) particles/\( ml \), because these concentrations had been shown to saturate the binding protein (Bolander & Blackstone, 1990).

Lysosomotropic amines localize to lysosomes and neutralize the acid in these organelles. If lowering the pH were necessary for cell penetration, these agents should prevent cell entry. Because ammonium chloride, chloroquine and methylamine had been successfully used in rabbit mammary glands (Houdebine, 1980), they were good candidates for testing in the mouse system. Unfortunately, ammonium chloride and chloroquine were so toxic that they completely blocked the prolactin induction of \( \alpha \)-lactalbumin in mammary explants (Table 1); only methylamine did not significantly interfere with mammary differentiation. Therefore, the effect of this latter agent on MMTV infection could be evaluated without fear that any results might be due to non-specific cytotoxicity. MMTV infection was accomplished by exposing mammary epithelium from MMTV mice to virus in culture and then hormonally inducing the tissue to express the virus. Epithelium exposed to methylamine produced only 48% of the MMTV RNA that control tissue did (1.13 ± 0.05 vs 2.33 ± 0.10 MMTV RNA/100 mg tissue, respectively), demonstrating that reducing the acidification of endosomes does indeed impair MMTV infection in mammary epithelium.

Yet, isolated gp52 failed to induce receptor internalization; one possible explanation for this failure is that other viral components are also involved in cell penetration. To test this hypothesis, intact MMTV was compared to the isolated gp52 with respect to its ability to induce internalization. Fig. 1 shows that, in the presence of intact MMTV, the number of binding proteins at the cell surface decreased with time, while that in the microsomes increased. This result demonstrated that the intact MMTV possessed all of the components required for internalization. On the other hand, neither cell surface nor microsomal receptors changed in the presence of isolated gp52, confirming earlier observations (Bolander, 1994). Finally, methylamine only slightly reduced the internalization of the receptor in response to MMTV; this suggested that methylamine is acting at the level of endosomal acidification and not affecting internalization proper.
Previous studies from this laboratory also demonstrated a correlation between the phosphorylation and internalization of MMTV receptors. Therefore, it was of interest to determine if intact MMTV could stimulate this modification in its binding protein. After 5 min exposure to isolated gp52, the amount of radioactive phosphate incorporated into the binding protein was not any greater than in controls (734 ± 56 vs 744 ± 74 c.p.m./100 mg tissue, respectively). In contrast, MMTV doubled receptor labelling (1476 ± 67 c.p.m./100 mg tissue), further strengthening the association between receptor phosphorylation and internalization.

There are three conclusions that can be drawn from this study: first, some lysosomotropic amines are toxic in certain systems. For example, in rabbit mammary explants none of these agents impaired lactose synthesis, although ammonium chloride did reduce DNA synthesis (Houdebine, 1980). In contrast, ammonium chloride did not affect cell number in primary cultures of mammary epithelial cells, although chloroquine was toxic in this system (Edery et al., 1989). In the mammary explant system, only methylamine proved safe (Table 1). Such discrepancies may be explained by differences in species (rabbit vs mouse), cultures (cells vs explants) or parameters measured (growth vs differentiation). Therefore, before any chemical is used in a new system, it should be tested for untoward side-effects.

The second conclusion is that MMTV requires internalization and acidification for successful infection of normal mammary epithelium; the third is that gp52 alone is insufficient to induce this internalization. What other factor(s) might be involved with MMTV cell entry? One factor may be another component of the envelope. For example, in Moloney murine leukaemia virus, the cellular receptor binding component, gp70, is anchored in the viral envelope by a second protein, p15E, and both are required for cellular penetration (Andersen, 1994). A similar subunit organization exists in MMTV where the receptor binding protein, gp52, is anchored in the envelope by gp36 (Dickson & Peters, 1983); it is possible that gp36 contributes to the infection process. Other examples can be found in members of the herpesvirus family where cellular attachment is biphasic: the initial attachment occurs between the viral protein gC and cell surface heparin; this is followed by the binding of gD to a second cellular receptor (Compton et al., 1993; Karger & Mettenleiter, 1993; McClain & Fuller, 1994).

Another factor in internalization may be aggregation: the viral envelope has a high density of gp52 on its surface. Interactions with the cellular receptor would naturally produce aggregation, which has been shown to be important for the function and metabolism of other receptors (Mohammadi et al., 1993; Rui et al., 1994). Free gp52 would not have this effect. However, anti-

bodies to the MMTV binding protein also do not induce internalization (data not shown), although they should produce clumping of the receptors because of their bivalent nature. If aggregation is important, it may depend on a special geometry unique to the viral envelope.

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References


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